ISSN 0704-3716

Canadian Translation of Fisheries and Aquatic Sciences

No. 5573

A simple and rapid method for measuring K value, a fish freshness index

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Original title: Gyorui sendo hantei kosu, K chi no kani sokuteiho no kairyo

In: Nihon Suisan Gakkaishi/Bull. Japn. Soc. Sci. Fish. 50(2): 263-267, 1984.

Original language: Japanese

Available from:
Canada Institute for Scientific and Technical Information
National Research Council
Ottawa, Ontario, Canada K1A 0S2

1992

16 typescript pages

Secretary of State / Secrétariat d'État

MULTILINGUAL SERVICES DIVISION – DIVISION DES SERVICES MULTILINGUES

TRANSLATION BUREAU BUREAU DES TRADUCTIONS

LIBRARY IDENTIFICATION – FICHE SIGNALÉTIQUE

Translated from - Traduction de	Into - En
Japanese	English

Author - Auteur

Hitoshi UCHIYAMA and Kiyonari KAKUDA

Title in English or French - Titre anglais ou français

A simple and rapid method for measuring K value, a fish freshness index

Title in foreign language (Transliterate foreign characters)
Titre en langue étrangère (Transcrire en caractères romains)

Gyorui sendo hantei kosu, K chi no kan'i sokuteiho no kairyo

Reference in foreign language (Name of book or publication) in full, transliterate foreign characters.
Référence en langue étrangère (Nom du livre ou publication), au complet, transcrire en caractères romains.

Nihon Suisan Gakkaishi

Reference in English or French - Référence en anglais ou français

Bulletin of the Japanese Society of Scientific Fisheries

Publisher - Éditeur	DATE OF PUBLICATION / DATE DE PUBLICATION			Page Numbers in original / Numéros des pages dans l'original
	Year / Année	Volume	Issue No. / Numéro	263-267
Place of Publication / Lieu de publication	1984	50	2	Number of typed pages / Nombre de pages dactylographiées: 15

Requesting Department / Ministère-Client: DFO

Translation Bureau No. / Notre dossier nº: 3850572

Branch or Division / Direction ou Division: Dr. R. Botta, NW Fisheries Centre
P.O. Box 5667, St. John's, Newfoundland
A1C 5X1

Translator (Initials) / Traducteur (Initiales): GN

Person requesting / Demandé par: Dr. R. Botta

SEP 15 1992

Your Number / Votre dossier nº:

Date of Request / Date de la demande: 92-04-08

Canada

SEC 5-111 (84-10)

Department of the Secretary of State of Canada | Secrétariat d'État du Canada

TRANSLATION LETTERHEAD - PAPIER À EN-TÊTE

MULTILINGUAL TRANSLATION DIRECTORATE
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DIRECTION DE LA TRADUCTION MULTILINGUE
BUREAU DE LA TRADUCTION

Originator File No. Référence du demandeur	Department - Ministère	Division - Branch/Direction	City - Ville
	DFO	Scientific Publications	Ottawa
Translation Request No. N°. de la demande de traduction	Language - Langue	Translator (Initials) - Traducteur (Initiales)	SEP 15 1992
3850572	Japanese	CN	

(Received May 16, 1983)

A Simple and Rapid Method for Measuring K Value, A Fish Freshness Index

Hitoshi UCHIYAMA and Kiyonari KAKUDA*2

Attempts were made to improve the method for measuring K value, a fish freshness index which is defined as the ratio of the sum of inosine (HxR) and hypoxanthine (Hx) to that of ATP and its autodegradation products in *postmortem* fish muscle.

Ion exchange resin (Bio-Rad, AG 1-X4, 100–200 mesh, Cl^- type) was washed with acetone and activated with 1 N NaOH and 1 N HCl. A column (0.6×5 cm) of the resin was washed with 10 m*l* of solution A (1.9 g sodium tetraborate, 1.2 g NaCl, 22.5 m*l* 1 N HCl, 10 m*l* 1 M ethylenediamine per 1 *l*, adjusted to pH 7.5).

The muscle was minced, extracted with cold perchloric acid and neutralized to pH 6.5–6.8 with caustic potassium solution. The column was charged with the extract equivalent to 0.1–0.2 g muscle, and washed with deionized water. HxR and Hx were eluted with solution A (elute A) while IMP, AMP, ADP and ATP were eluted with solution B (17.4 g NaCl and 150 m*l* 1 N HCl per 1 *l*) (elute B).

Elutes A and B were made up to 50 m*l* and 25 m*l* respectively and measured for absorbance at 250 nm to calculate K value by the following formula:

$$K(\%)=\frac{E_{250\,nm}A}{E_{250\,nm}A+E_{250\,nm}B\times 1/2}\times 100$$

This simple method was satisfactorily applied to squid muscle as judged by the standard deviation of K value, 0.12–0.16.

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In this report the following abbreviations are used. TVB-N: volatile basic nitrogen; TMA-N: trimethylamine nitrogen; ATP: adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate; IMP: inosinic acid; HxR: inosine; Hx: hypoxanthine; PCA: perchloric acid.

The fact that K value,[1] a fish freshness index, is of more practical use in terms of judging freshness than measurements based on bacterial degradation products (TVB-N, TMA-N) was discussed in detail in previous reports.[2,3] Since then, the significance of K value measurement has been recognized by many researchers. For example, it has been used in clarifying[4] the freshness of tuna and its browning phenomenon due to metmyoglobin formation.

Recently, UTSUKI et al.*3 analyzed the K value of many tropical, subtropical and temperate zone fish species, and have suggested that there is a correlation between changes in K_D,[5] a protein degeneration speed index, and K value, and that fish species with a high K_D have a rapid rise in K value. In this way, interesting information has been presented one after another. Also, the fact that, in frozen fish, it is far more practical, in terms of quality judgment, to measure the K value than measure the protein degeneration, was made clear in a previous report.[6]

On the other hand, with recent developments in analytical equipment, attempts at automatic analysis of ATP related compounds in fish muscle using high speed liquid chromatography, and in turn to quicken the computation of K value, have been put forth by LEE et al.[7] And furthermore, using sensors based on enzyme electrodes, even the development of a freshness judging device[8] has come to be undertaken.

Meanwhile, the Fisheries Administration Department of the Fishery Agency has, on a five year plan from 1982, commenced a

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*3 UTSUKI, Teruhiro, et al.: Summary of Lectures at the 1983 Spring Convention of the Japanese Society of Scientific Fisheries, p. 231 (1983).

distribution improvement experimental project[9,10] based on indicating the quality of fresh fish. The intent is to stipulate the freshness by K value and indicate that value on a label to provide consumers with good quality fish. In order to attain this sort of goal, the development of a simple quantification method that does not cost too much becomes a prerequisite. With the thought of wishing to respond to this requirement, improvements were made to the simple column chromatographic method in previous reports[11,12] and attempts were further made to quicken measurement and increase accuracy. A report on that is presented below.

Method of experiment

Samples. The Hx, HxR, IMP, AMP, ADP and ATP used were those of Sigma Co. and Merck Co. Using a 10 mM concentration, they were stored at -20°C and diluted for use.

Preparation of muscle extract solution. The sample used was Ommastrephes sloani pacificus (those caught in fixed shore nets at Misaki, Miura-shi, Kanagawa-ken as well as commercial products sold in Tokyo, Chiba and Hyogo Prefectures). As in the previous reports,[11,12] KOH neutralized solutions of PCA extracted solutions were prepared and subjected to column chromatography as described below.

Column. A column with an inner diameter of 0.6 cm and a length of approximately 15 cm was used. Details on this were provided along with figures and photographs in the previous reports[11,12] so they will be omitted here.

Ion exchange resin. A Bio-Rad, AG 1-X4, 100-200 mesh, Cl^- type was used. After activating it with acetone, 1 N NaOH and 1 N

HCl in that order while washing with deionized water each time, it was once again thoroughly washed with deionized water and stored in a refrigerator. This preparation, although differing in resin, was exactly the same as in the case of Dowex in the previous reports.[11,12]

Eluant. Solution A: 1.9 g of borax, 1.2 g of NaCl, 22.5 mL of 1 N HCl and 10.0 mL of 1 M ethylenediamine were dissolved to make up 1 L. At this mixture ratio, the pH is close to 7.5, but it is desirable to adjust* it anew to exactly 7.5. Solution B: 17.4 g of NaCl and 150 mL of 1 N HCl were made up to a set volume of 1 L.

Fractionation procedure. After packing the resin to a height of approximately 5 cm, approximately 10 mL of solution A are poured in. Putting the extracted sample (equivalent to 0.1-0.2 g of muscle) into a 20 mL capacity beaker, this is adjusted to pH 9.4 with ammonia water (approximately 1 drop of 1 M ammonia water, TB test paper). Charging the column with this, the beaker is washed with deionized water adjusted to pH 9.4 with ammonia water, which is added to the column.

First, 20 mL of deionized water are poured in, and the non-adsorptive ultraviolet absorbance substances are removed in the columns other than for ATP related compounds. Next, approximately 45 mL of solution A are put in a separating funnel which is connected to the column. Collecting the eluant into a 50 mL measuring flask, just to make sure, a small amount of solution A is once again added to the column to make up 50 mL. By means of this

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* (It is convenient to use Merck Co.'s trichromatic pH strip, product No. 9533.)

procedure Hx and HxR are eluted. Next, adding approximately 20 mL of solution B to a separating funnel and collecting this in a 25 mL measuring flask, a fixed volume is made up in the same way as in the case of solution A. By means of this procedure IMP, AMP, ADP and ATP are eluted. Shaking well the fractions of solution B and solution A and measuring their respective absorbance (250 nm), the K value was computed by the following equation.

$$K(\%) = \frac{E_{250\,nm}A}{E_{250\,nm}A + E_{250\,nm}B \times 1/2} \times 100$$

Experimental results

Elution volume of Hx. Fig. 1 shows a profile of the eluted quantity of Hx by solution A when 2 mL each were fractionated at a speed of 2 mL/min using a chromatographic mini-pump and fraction collector. Using 1 μmol of the sample, at first it was eluted with approximately 20 mL of deionized water, but no Hx was eluted whatsoever. When we switched from deionized water to solution A, with several mL the elution of Hx started, and within 20 mL it was completed.

Elution volume of HxR and AMP. Fig. 2 shows a profile of the eluted quantity of HxR by solution A and AMP by solution B using 1 μmol each of HxR and AMP. With deionized water the elution of HxR could not be discerned, but with solution A the elution began and it was completed with a volume of approximately 25 mL. Also, as can be seen in the figure, AMP did not get eluted by solution A but it did when we switched to solution B. It was completed with an elution quantity of approximately 16 mL.

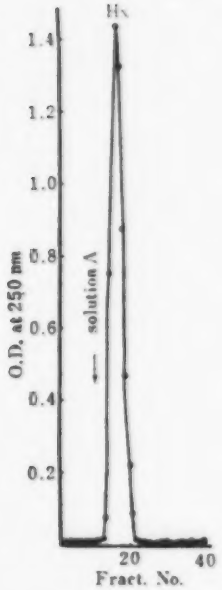

Fig. 1. Chromatographic profile on an ion exchange resin column of hypoxanthine (Hx) when eluted by solution A.

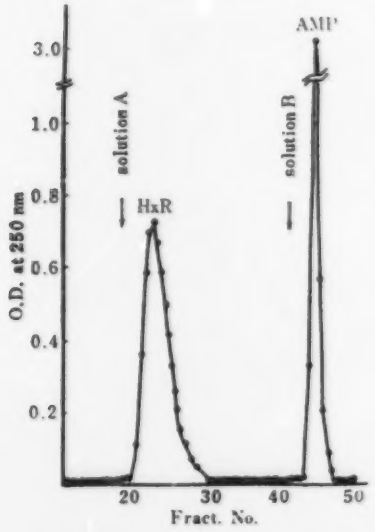

Fig. 2. Chromatographic profile on an ion exchange resin column of a mixture of inosine (HxR) and AMP when eluted by solutions A and B respectively.

Recovery rate of Hx, HxR and AMP. The absorbance of Hx at 1 mM, pH 7.0, 250 nm is a theoretical value[13] of 10.70, but in the case of the experimental value in Fig. 1, 10.59 was obtained, and its recovery rate became 99.0%. Similarly, the theoretical value for HxR under the same conditions is 12.43, but in the case of the experimental value in Fig. 2, 12.30 was obtained, and the recovery rate became 99.0%. Again, with AMP the theoretical value was 12.07 (pH 2) and the experimental value (Fig. 2) 11.97, with the recovery rate becoming 99.2%. In every case the values obtained posed no problems for the measurement of K value.

Elution quantity by solution A for Hx and HxR mixture. Fig. 3 shows a profile of the elution quantity by solution A using a mixture of 1 μmol each of Hx and HxR. With 50 mL of solution A the recovery rate for both compounds became 99.3%, reconfirming that 50 mL is sufficient for fractionating a mixture of Hx and HxR for the measurement of K value.

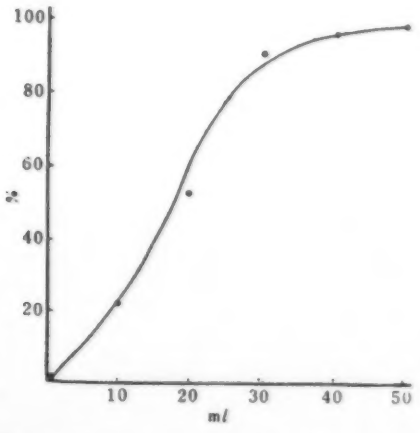

Fig. 3. Recovery of a mixture of 1 μmol each of inosine (HxR) and hypoxanthine (Hx). Eluant: solution A.

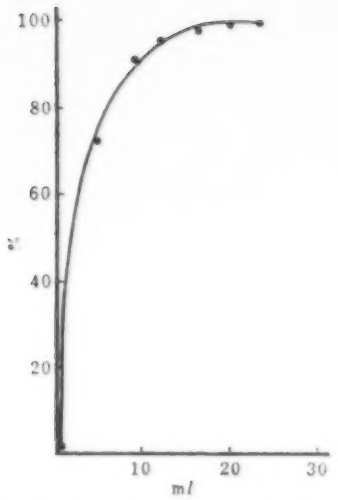

Fig. 4. Recovery of a mixture of 1 μmol each of IMP, AMP, ADP and ATP.
Eluant: solution B.

Elution quantity by solution B for IMP, AMP, ADP, ATP mixture. Similarly, Fig. 4 shows a profile of the elution quantity by solution B using a mixture of IMP, AMP, ADP and ATP (1 μmol each). With 12 mL the elution of all compounds yielded a recovery rate of 100.5%, revealing that fractionation with 25 mL is ample.

Standard deviation and coefficient of variation when the same sample is analyzed. While the purpose of measuring the K value of squid will be discussed later, errors are more prone to occur than in fish meat. Accordingly, as such samples, just-caught Ommastrephes sloani pacificus with a low K value and commercially sold ones with a high value were used to verify the accuracy of this measurement method.

Table 1 shows the results where samples No. 1 and 2 were measured, with the same sample being measured 10 times each. The standard deviations obtained for the two samples were 0.16 and 0.12 while the coefficients of variation obtained were 2.76 and 0.17.

Table 1. Standard deviation and coefficient of variation of *K* values of two squid samples

No.	Sample No. 1*1	Sample No. 2*2
	K value (%)	
1	5.8	68.5
2	5.7	68.8
3	6.1	68.7
4	5.7	68.4
5	5.9	68.6
6	5.8	68.8
7	5.5	68.5
8	6.0	68.7
9	5.8	68.4
10	5.7	68.5
Mean	5.8	68.6
S.D.*3	0.16	0.12
C.V. (%)*3	2.76	0.17

*1 Killed immediately after being caught.
*2 Bought at a retail shop.
*3 S.D. and C.V.: Standard deviation and coefficient of deviation respectively.

Table 2. *K* values of ten commercial samples of frozen squid

Sample No.	*K* value (%)
1	67.1
2	71.9
3	83.9
4	67 4
5	67.3
6	73.8
7	68.7
8	72.0
9	68.8
10	60.5

K value of commercially sold Ommastrephes sloani pacificus. Table 2 shows the results where Ommastrephes sloani pacificus sold commercially in Tokyo, Chiba and Hyogo Prefectures were randomly sampled and their K values measured. As can be seen in the table, the K values are abnormally high compared to those just caught in Table 1, and the freshness as viewed from these analyzed values was judged to be exceedingly poor.

Discussion

The K value[1)] is a ratio of the total ATP related compounds at E_{250nm} to the Hx + HxR fraction at the same wavelength in fish muscle, so there is a need to accurately fractionate and quantify these two fractions. For this reason, in the previous reports[11,12)] Hx + HxR had been fractionated from nucleotides such as AMP using 0.001 N HCl. Measurements are possible even with this, but if Hx + HxR were fractionated with a slight alkalinity, there would be absolutely no risk of the elution of nucleotides, and the fractionation would be more stable. Also, by preparing solution A

as stated in the experimental method it becomes pH 7.5 as is, and moreover because sodium tetraborate has been added, it has a buffer action and the maintenance of a slight alkalinity in the column is stabilized.

The foregoing points may be said to be conditions to make special note of in the application of K value to judging the freshness of squid. This is because the K value has been primarily applied to judging the freshness of fish, but recently there has been a demand industrially to apply it to squid and shrimp as well. The main reason is that TVB-N discussed by OKUDA[14] does not serve as an index of freshness, and there is a demand to judge the biochemical freshness or so-called "liveliness." In point of fact, in squid, between those with a low K value (5.5-6.1%) and high K value (60.5-83.9%) there was only a difference of 2-3 mg/100 g or so in TVB-N. Consequently, in the present state of things there would seem to be no alternative but to apply K value. Moreover, since the aforementioned distribution improvement experimental project of the Fisheries Administration Department of the Fishery Agency was begun, the K value of squid has been displayed and it has come to be recognized that those with a low value have a high degree of freshness.

Now, as SAITO et al.[15] have made clear, in squid, unlike fish, IMP as an ATP degradation product does not form, and AMP decomposes into HxR by deaminization and such. Consequently, AMP must be given importance as an indicator for judging freshness. However, as discussed in a previous report,[16] with a Cl^- type ion exchange resin, HxR and AMP are closer in elution position relative to IMP,

necessitating the precaution that pH and NaCl concentration must always be accurately maintained for their fractionation. The present improved method does not have to be concerned with this point because Hx + HxR would be fractionated with a slight alkalinity, making the measurement simple and accurate.

Also, in squid there are more ultraviolet absorbance substances other than ATP related compounds than in fish. These are removed by deionized water, but by making the column slightly alkaline beforehand, as can be seen in Fig. 1 and Fig. 2 they can be removed by deionized water with absolutely no risk of the elution of Hx and HxR.

The Bio-Rad resin is more uniform in grain size than Dowex used in the previous reports,[11,12] and moreover a coarse grain size of 100-200 mesh was used, so there is the advantage that the elution time gets shortened.

The Cl^- concentration of solution B is somewhat higher than the Cl^- concentration required for ATP elution (0.13 M Cl^-). Consequently, elution is rapid. Also, while this would be a matter of course, IMP, AMP and ADP which are elutable at a lower Cl^- concentration than for ATP, can be eluted right away by solution B, the volume of which is ample at 25 mL, and from this perspective as well the quickening of measurement has become possible.

As shown in Table 1, the K values measured 10 times with the same squid samples were virtually uniform, and the standard deviation regardless of whether the K value was high or low was 0.12-0.16. Thus, in terms of judging freshness, the accuracy may be said to be satisfactory.

Table 2 gives an example of the results of the measurement of K value of commercially sold frozen Ommastrephes sloani pacificus. The values are unexpectedly high, and many are poor in freshness. As can be seen in Table 1, the K value just after squid are caught is 5.8%, so these abnormally high K values are considered to be due to mismanagement of freshness control in the handling stages of the distribution process. The reality of this situation is currently being examined using this improved method.

As above, this improved method is simple in procedure, and if a column and separating funnel were standardized and used, K value measurements of 30 samples or so per day will be easy. The Bio-Rad resin is also extremely cheap compared to resins for high speed liquid chromatography. Moreover, the reproduction process is simple, so that from an economic aspect as well, it is thought this method can be utilized extensively in K value measurements of fish and squid.

Summary

Improvements were made to the simple method of measuring K value, a fish freshness index, given in a previous report, and simplification and measurement accuracy were further enhanced.

The K value is a ratio of the total ATP related compounds at E_{250nm} to the hypoxanthine (Hx) and inosine (HxR) fraction at the same wavelength in fish muscle, so there is a need to accurately fractionate and quantify the two fractions. Packing Bio-Rad resin (AG 1-X4, 100-200 mesh Cl^- type) into a column with an inner diameter of 0.6 cm and a height of approximately 15 cm, Hx and HxR are eluted with solution A (1.9 g of borax, 1.2 g of NaCl, 22.5 mL

of 1 N HCl and 10 mL of 1 M ethylenediamine added to deionized water to make up 1 L, pH 7.5). Solution A is slightly alkaline (pH 7.5), so the fractionation of Hx and HxR was possible with no risk of the elution of nucleotides such as AMP. Next, using solution B (17.4 g of NaCl and 150 mL of 1 N HCl added to deionized water to make up 1 L), IMP, AMP, ADP and ATP are fractionated in the same fraction, and the K value was computed from E_{250nm} of solutions A and B respectively.

The Bio-Rad resin is more uniform in grain size than Dowex in the previous report, and moreover a coarse grain size of 100-200 mesh was used, so the elution was quickened. Also, the elution volume for nucleotides such as AMP decreased because the Cl^- concentration of the eluant was raised, so the fractionation time was shortened by that much.

The K value has been primarily used in judging the freshness of fish, but this method has turned out to be applicable to judging the freshness of squid as well. The reason is that it was possible to accurately fractionate AMP which is an indicator of the freshness of squid. The standard deviation of K values measured 10 times with the same squid samples was 0.12-0.16, so this method is thought to be utilizable extensively as a simple method of measuring practical K values.

Now, it was not unusual for commercially sold frozen squid to indicate a K value of around 68%, and the freshness as viewed from this value was judged to be exceedingly poor. It is surmised to be attributable to mismanagement in freshness control in the distribution process.

We would like to express our sincere gratitude to Chief Researcher Fumiaki UDA of the Nomura Bioscience Research Institute and to Dr. Shigeo EHIRA of the Tokai Regional Fisheries Research Laboratory for their valuable opinions in the course of conducting this experiment.

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